(Anti-)TNF alpha matters in rheumatoid arthritis

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Chapter 5

Analysis of Apoptosis in Peripheral Blood and Synovial Tissue Very Early after Initiation of Infliximab Treatment in Rheumatoid Arthritis Patients.

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ABSTRACT

Objective.
Infliximab treatment results in a decrease in synovial cellularity as soon as 48 hours after initiation of therapy in patients with rheumatoid arthritis (RA). We investigated whether infliximab induces apoptosis within the first 24 hours after infusion.

Methods.
Apoptosis was detected by flow cytometry in blood drawn directly before, and 1 hour, and 24 hours after completing the infliximab infusion from 21 patients. Synovial tissue obtained before and 1 hour (n = 5) or 24 hours (n = 5) after initiation of therapy was used to detect active caspase 3 by immunohistochemistry and apoptosis by TUNEL assay and electron microscopy (EM). In addition, plasma levels of nucleosomes (apoptosis) and C4b/c (complement activation) were measured.

Results.
There were no signs of apoptosis induction in peripheral blood monocytes or lymphocytes after infliximab treatment. Circulating lymphocyte counts were increased within 1 hour (P < 0.05) after infusion. There was no definite evidence of apoptosis induction in the synovium except in one patient 24 hours after the infliximab infusion. Consistent with these results, there was no increase in nucleosome levels nor were there signs of complement activation.

Conclusion.
These data support the notion that the rapid decrease in synovial cellularity observed after initiation of anti-TNF antibody therapy cannot be explained by apoptosis induction at the site of inflammation. It is tempting to speculate that the striking effects on synovial inflammation may be explained by other mechanisms, like decreased migration towards the synovial compartment and reduced retention in the inflamed synovium.
Disease control has been greatly improved by the use of tumor necrosis factor (TNF) blockade in patients with rheumatoid arthritis (RA) (1,2). Insight into the mechanism of action may help to understand the effects of different TNF antagonists in various immune-mediated inflammatory diseases, but could also lead the way to the identification of new therapeutic strategies. However, the mechanisms by which anti-TNF therapy exerts its effect are still not completely understood (3).

A common observation in the treatment of immune-mediated inflammatory diseases with TNF antagonists is the rapid reduction in cellularity at the site of inflammation, but the relative roles of apoptosis, cytotoxicity, as well as reduced cell influx and enhanced cell egress still remain to be elucidated. Previous work has shown a significant decrease in synovial inflammation 48 hours, 14 days, and 28 days after the first dose of infliximab in RA patients (4-6), but detailed analysis including TUNEL assays and electron microscopy showed no evidence of apoptosis induction that could explain the swift decrease in synovial cellularity (4). These results were confirmed in patients with psoriatic arthritis, using a similar study design (7). It was critical in these studies to include electron microscopy as a gold standard for the detection of apoptotic cells in tissue, since several studies have shown only a low frequency of apoptotic cells in rheumatoid synovial tissue despite the presence of fragmented DNA as shown by TUNEL assays, which can be explained by impaired apoptosis in the RA synovium [reviewed in (8)]. It has been suggested, however, that after more prolonged treatment increased apoptosis could occur (9), but this may be a secondary phenomenon rather than a direct pro-apoptotic effect of TNF blockade (3).

Although there is no evidence that the reduced synovial cell infiltrate observed 48 hours after initiation of infliximab therapy can be explained by apoptosis induction at the site of inflammation, we cannot exclude the possibility that apoptosis occurs as a direct effect of treatment even earlier after infusion and becomes undetectable after 48 hours due to rapid clearance of apoptotic cells by phagocytosis (10). Support for this notion comes from a study showing an increase in plasma nucleosomes (generated during apoptosis) in 5 out of 8 RA patients 24 hours after infliximab infusion; this increase could even be detected as early as 2 hours after the infusion (11). Consistent with the hypothesis that apoptosis may be detectable at 24 hours after infliximab infusion, there was an increase in TUNEL positive cells in the lamina propria T cells of the gut in patients with Crohn’s disease at that time point (12). An alternative explanation for the reduced synovial cell infiltrate at 48 hours without signs of apoptosis at the site of inflammation could be that apoptosis induction occurs in the peripheral blood rather than in the synovial compartment, leading to reduced cell influx.
Therefore, we studied apoptosis induction in the synovium of RA patients at 1 hour and 24 hours, respectively, after initiation of infliximab treatment. In addition, we investigated apoptosis induction in peripheral blood monocytes and lymphocytes at these same time points.

**PATIENTS AND METHODS**

**Patients.**

Twenty-one RA patients (13) with a Disease Activity Score (DAS28) (14) of at least 3.2 were included in the study. All patients received infliximab 3 mg/kg intravenously in combination with methotrexate in a stable dose for at least 8 weeks. Sixteen patients received their first infusion (group 1) and 5 additional patients (group 2) were already on infliximab treatment (3 mg/kg intravenously) for 11 months, and had their last infusion 8 weeks before. All 21 patients underwent blood sampling before, 1 hour, and 24 hours after infusion. The infusion was completed in 1 hour. An arthroscopy was performed before and 1 hour (n = 5) or 24 hours (n = 5) after initiation of infliximab therapy in anti-TNF naïve patients (belonging to group 1). Approval for this study was obtained from the institutional ethics review committee at the Academic Medical Center/University of Amsterdam and all participants gave their written informed consent.

**Flow cytometry.**

Fresh blood samples were retrieved from patients before (T=0), 1 hour (T=1) after and 24 hours (T=24) after completion of the infliximab infusion. We studied the peripheral blood mononuclear cells (PBMCs) on the day of collection without additional in vitro stimulation or infliximab incubation.

Erythrocytes were lysed with lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, PH 7.4) and kept on ice for 20 minutes. The remaining cells were washed twice with the ice cold isotonic lysis buffer at 1700 rpm for 5 minutes at 4°C and diluted to a concentration of 2.0 x 10⁶ cells/ml. Next, 5.0 x 10⁵ cells (volume 250µl) were incubated with surface markers for 30 minutes and kept on ice. Surface markers included 5 µl FITC-labeled anti-human CD14 (M5E2, Becton Dickinson (BD), San Jose, CA) to study monocytes and 5 µl FITC-labeled anti-human CD3 (SK7, BD) to study T-lymphocytes. To prevent non-specific binding 10 µl normal human serum was added. Cells were washed in 3 ml ice-cold FACS-buffer (BD) at 1700 rpm for 6 minutes at 4°C. Subsequently, the samples were incubated for 15 minutes on ice with 5 µl PE-labeled annexin V (BD), and 5 µl 7-Amino Actinomycin D (7AAD) (BD). The control samples were treated in the same manner as the patient samples. As a positive control PBMCs were incubated with different prednisone concentrations (6.25, 12.5, 25, 50, 100 and 200 mM) for 24 hours and subsequently incubated with annexin V and 7AAD. Analysis was conducted by flow cytometry (FACS Calibur).
The percentages CD14+ or CD3+ annexin V single positive cells were defined as early apoptotic cells and 7 AAD single positive cells as necrotic cells. Double positive cells were determined to be late apoptotic cells. The number of apoptotic cells was expressed as percentages of the total number of gated T-lymphocytes or monocytes.

Nucleosome ELISA.

The presence of mono- and oligonucleosomes in patient plasma drawn before as well as 1 hour and 24 hours after infliximab infusion was determined with a commercial quantitative sandwich-immunoassay (Cell death detection ELISA plus, Roche diagnostics, Mannheim, Germany). The assay was performed according to the manufacturer’s instructions. EDTA plasma was centrifuged at 3000g (4°C) for 10 minutes within 30 minutes after venipuncture and immediately stored at -80°C (15). All samples were analyzed in one procedure. Plasma instead of serum was used to avoid additional DNA release during the clotting process (16).

For confirmation nucleosome levels were also determined in 10 patients by a non-commercial nucleosome ELISA developed by Sanquin research. This ELISA used two anti-nuclear monoclonal antibodies (ANA-58 and ANA-60 both developed by Sanquin, Amsterdam, the Netherlands).

The assay was performed as described previously (17). As a standard culture supernatant of Jurkat cells (10⁶ cells/ml), which were cultured for a week, was used resulting in 100% apoptotic cells. One unit reflects the number of nucleosomes released by approximately 100 Jurkat cells. The detection limit of the assay was 5.0 U/ml. The same ELISA was repeated after a 2 time IgM rheumatoid factor neutralization step (1A14-22 IgM-RF neutralization kit, Imx systems; Abbott Laboratories, IL) to rule out false positive results due to the presence of rheumatoid factor.

Differential leukocyte counts.

An automated Coulter cell counter was used for total leukocyte counting (10⁹ cells/L) and the determination of differential cell percentages. Subsequently, absolute cell counts were calculated from the known percentages of monocytes, lymphocytes, and neutrophils before and after infliximab infusion. Cell counts were performed on fresh blood samples from 16 patients (11 from group 1 and 5 from group 2) collected in tubes containing EDTA.

Clinical chemistry.

Lactate dehydrogenase (LDH) and C-reactive protein (CRP) levels were determined in the same 16 patients by the certified routine clinical chemistry laboratory at the Academic Medical Center in Amsterdam. LDH levels (U/L) were monitored before, 1, and 24 hours after infliximab as a measure of cell lysis. CRP levels (mg/L) were measured at the same time points.
Complement activation.

Activation of the classical complement pathway was measured by an ELISA assay that measures the levels of C4b/c (activated C4). For the quantification of these complement activation products in EDTA plasma, a previously described ELISA was used (18). In short, a monoclonal anti-C4-1 antibody recognizing a neo-epitope on activated C4 was used as catching antibody. Biotinylated polyclonal rabbit anti-human anti-C4 antibody was used for detection. The ELISA was performed on ice. Aged human serum, containing a known amount of activated C4, was used for the calibration curve.

Synovial biopsy and immunohistochemical analysis of the cell infiltrate.

From 10 out of 21 RA patients (group 1) synovial biopsies were obtained by mini-arthroscopy of the knee joint. Five patients were biopsied before and 1 hour after completing the first infliximab infusion, 5 other patients were biopsied before and 24 hours after completing the first infliximab infusion; for obvious ethical reasons we could not perform 3 arthroscopies in the same patients during the 24 hour period. The arthroscopy, tissue sampling, and storage were performed as previously described (19). The cell infiltrate was characterized by immunohistochemical analysis using the following monoclonal antibodies: anti-CD55 (67: Serotec, Oxford, UK) for fibroblast-like synoviocytes (FLS), anti-CD3 (SK7, BD) for T cells, anti-CD68 (EBM11: DAKO, Glostrup, Denmark) as a pan–macrophage marker, anti-CD22 (CLB-B-ly/1,6B11, Central Laboratory of the Netherlands Red Cross Blood Transfusion service (Sanquin), Amsterdam, The Netherlands) for B cells, and anti-CD38 (HB-7; BD) for plasma cells. For the detection of adhesion molecules we used: anti-ICAM-1 (MEM111, Sanbio, Uden, Belgium) and anti-VCAM-1 (1G11B1, Sanbi, Uden, Belgium). Staining for cellular markers was performed using a 3-step immunoperoxidase method as described previously (20).

TUNEL assay and detection of active caspase 3.

Apoptosis of synovial cells was evaluated by terminal deoxy (d)-UTP Nick End Labeling (TUNEL), which detects DNA strand breaks induced by cell death. An in situ cell death detection assay (Roche Diagnostics, Mannheim, Germany) was used, according to the

Active caspase 3 was detected with an affinity purified rabbit anti-human cleaved caspase 3 antibody (Asp175; Cell Signaling Technology Inc, Danvers, MA). First, tissue was fixed in formalin for 5 minutes. Between all steps, the sections were washed with Phosphate Buffered Saline (PBS). Endogenous peroxidase activity was blocked using 0.1% sodium azide and 0.3% hydrogen peroxide in PBS. Irrelevant binding was blocked by normal goat serum diluted (1:10) in PBS. Staining was performed using a 2-step immunoperoxidase method. The primary rabbit anti-human antibody was incubated overnight followed by a 30 minute incubation with the rabbit EnVision+ system (K4009; DAKO). HRP activity was detected using hydrogen peroxide as
substrate and 3-amino-9-ethylcarbazole (AEC) as a dye (reddish color). As a negative control the
primary antibodies were omitted or irrelevant immunoglobulins were used.

Digital image analysis.

All coded sections were randomly analyzed by computer-assisted image analysis and the total
number of positive cells per square millimeter (counts/mm²) was determined. The images of
the high-power fields were analyzed using the Qwin analysis system (Leica, Cambridge, UK), as
described previously in detail (21).

Electron microscopy.

Chromatin condensation was evaluated by electron microscopy (EM) as a gold standard
for apoptosis detection in tissue. For this purpose small pieces of fresh synovial tissue were
immersed in Karnovsky’s fixative (Department of Pathology, AMC) and embedded in Epon
(Ladd Research Industries Inc, Burlington, VT). Thin sections were briefly pretreated with
diluted tannic acid for better visualization of extra cellular matrix components before treating
them with uranyl and lead according to standard procedures (22).

Statistical analysis.

Data were analyzed by non-parametric methods using the Wilcoxon signed ranks test to deter-
mine significant changes from baseline. Probability values < 0.05 were considered statistically
significant in a 2-tailed test. Values are expressed as the mean ± standard deviation (SD) or
median and range or interquartile range (IQR), whichever was appropriate. The calculations
were performed with SPSS 12.0.2 for Windows (SPSS, Chicago, IL).

RESULTS

Patients.

The group of 21 RA patients who were analyzed consisted of 12 women and 9 men. Most of
the patients were positive for IgM rheumatoid factor (71%) and anti-cyclic citrullinated peptide
antibodies (81%). Their mean age was 53 ± 14 years, and the median disease duration was 79
(range 18-504) months. See Table 1 for baseline patient characteristics.

Infliximab does not induce apoptosis of peripheral blood monocytes and lymphocytes.

All samples were analyzed for the presence of apoptotic cells. Figure 1 shows representative
flow cytometry results for CD14+ monocytes (Figure 1A) and CD3+ T lymphocytes (Figure 1B).
There was no statistically significant increase in the percentage of either early (annexin V single
positive) or late (annexin V and 7AAD positive) apoptotic peripheral blood monocytes and
T-lymphocytes within 1 or 24 hours after infliximab infusion. Flow cytometry data are shown in
Table 2. Importantly, no evident differences in results were observed for the 16 patients receiving infliximab for the first time compared with the 5 patients who had already been treated with infliximab for 11 months. One 24 hour sample was missing.

To exclude a false negative effect PBMCs were incubated for 24 hours with 6.25 mM or 200 mM prednisone, serving as a positive control. This resulted in induction of early apoptosis in 11.6% and 16.1% of the cells, respectively. Late apoptosis was detected in 2.2% and 13.4% of the cells, respectively (Figure 1 C-D).

Infliximab treatment does not induce an early increase of plasma nucleosomes.

Circulating nucleosome levels in EDTA plasma were below the detection limit of the Cell death detection ELISA plus (Roche Diagnostics) both before, and 1 and 24 hours after infliximab treatment, although the laboratory positive controls worked well. These results were confirmed using an independent test, the Sanquin anti-nucleosome ELISA.

Lymphocytosis and unaltered numbers of circulating monocytes and neutrophils after infliximab infusion.

The absolute number of circulating lymphocytes increased rapidly within 1 and 24 hours after treatment ($P=0.044$ and $P=0.013$, respectively). There was no clear-cut change in circulating monocytes or neutrophils. Peripheral blood cell counts are shown in Table 2.

Infliximab treatment does not induce complement activation or cell lysis.

Having shown that there was no reduction of circulating leukocytes, we also measured LDH levels as a reflection of cell lysis. There was no statistically significant increase in LDH levels after treatment, suggesting that no major cell lysis occurred within the first 24 hours after the

**Table 1. Baseline patient characteristics.**

<table>
<thead>
<tr>
<th>Demographics</th>
<th>N=21</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53 ± 14</td>
</tr>
<tr>
<td>Female (%)</td>
<td>12 (57)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease status</th>
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</thead>
<tbody>
<tr>
<td>Disease duration (months)</td>
<td>79 (18-504)</td>
</tr>
<tr>
<td>Erosive disease (%)</td>
<td>16 (76)</td>
</tr>
<tr>
<td>Rheumatoid factor positive (%)</td>
<td>15 (71)</td>
</tr>
<tr>
<td>Anti-CCP positive (%)</td>
<td>17 (81)</td>
</tr>
<tr>
<td>DAS28</td>
<td>5.8 ± 1.0</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>35 (8-79)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>24 (3-103)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug treatments</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate (mg/wk)</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Receiving corticosteroids (%)</td>
<td>5 (24)</td>
</tr>
<tr>
<td>Prednisone (mg/day)</td>
<td>10 ± 0</td>
</tr>
</tbody>
</table>

* Mean values ± SD, median and range or percentages are shown. DAS28 = disease activity score in 28 joints, ESR= erythrocyte sedimentation rate.
TNF blocking therapy and apoptosis induction

C-reactive protein levels did not show a clear cut change after 24 hours. Furthermore, C4b/c levels, the activation product of classical complement pathway, did not increase after infliximab infusion. Thus, there were no signs of complement activation or cell lysis (see Table 2.)

Figure 1. Cells stained with FITC-labeled anti-CD14 to identify monocytes (A), FITC-anti-CD3 to identify lymphocytes (B). Annexin V-PE and 7-AAD were used to detect apoptotic cells. Data are representative of experiments performed in 21 patients. Viable cells are found in the lower left quadrant, early apoptotic cells would be found in the lower right quadrant, and dead or late apoptotic cells are found in the upper right quadrant. Necrotic cells would be in the upper left quadrant. There was no increase in the percentage of circulating apoptotic monocytes or lymphocytes early after initiation of infliximab treatment. Healthy donor PBMCs incubated for 24 hours with prednisone were used as a positive control. Prednisone 6.25 mM induced 11.6% early apoptosis as seen in the lower right quadrant (LRQ) (C) and prednisone 200 mM induced 16.1% early apoptosis (D). An increase in necrosis is seen in the upper right quadrant at the 200 mM prednisone dose.
Chapter 5

Decreased numbers of synovial T cells and macrophages 24 hours after the infliximab infusion. There was no decrease in synovial cell populations one hour after the initiation of infliximab treatment. However, 24 hours after the infliximab infusion there was a decrease in the median number of synovial CD68+ sublining macrophages: 739 cells/mm² (IQR 14-1813) before compared to 280 cells/mm² (IQR 36-1609) after treatment. Similarly, the median number of CD3+ T cells was reduced from 306 cells/mm² (IQR 0-589) before to 27 cells/mm² (IQR 3-760) 24 hours after treatment. There was no decrease in the median number of synovial B cells (median 254 cells/mm², IQR 69-1065, 24 hours after treatment). The differences did not reach statistical significance, presumably due the relatively small number of patients. Consistent with these data, we have previously shown a marked reduction in synovial T cells and macrophages 48 hours after initiation of infliximab treatment in an independent group of patients (4).

Synovial apoptosis cannot explain the decreased cellularity early after infliximab treatment.

One hour after infliximab treatment there was a minor increase in the number of TUNEL+ cells in 2 patients and a decrease in the other 3 patients (Figure 2A). A slight increase in active caspase 3 + cells was seen in 3 out of 5 patients and a decrease in 2 patients (data not shown). Consequently, there was no clear cut change in TUNEL+ or active caspase 3 + cells 1 hour after the infliximab infusion.

Table 2. Percentage of apoptotic cells, absolute cell counts, markers of cell lysis and complement activation in relation to infliximab treatment.

<table>
<thead>
<tr>
<th>Flow cytometry analysis (N=21)</th>
<th>T=0</th>
<th>T=1 hr</th>
<th>Wilcoxon signed ranks test</th>
<th>T=24 hrs</th>
<th>Wilcoxon signed ranks test</th>
</tr>
</thead>
<tbody>
<tr>
<td>% early apoptotic CD14+ Monocytes</td>
<td>0.77 (0.40-1.31)</td>
<td>0.81 (0.34-1.54)</td>
<td>ns</td>
<td>0.95 (0.57-1.51)</td>
<td>ns</td>
</tr>
<tr>
<td>% late apoptotic CD14+ Monocytes</td>
<td>7.65 (5.39-15.18)</td>
<td>5.91 (2.54-10.88)</td>
<td>ns</td>
<td>6.63 (3.84-9.53)</td>
<td>ns</td>
</tr>
<tr>
<td>% early apoptotic CD3+ Lymphocytes</td>
<td>0.61 (0.24-1.75)</td>
<td>0.42 (0.26-1.05)</td>
<td>ns</td>
<td>0.64 (0.45-1.36)</td>
<td>ns</td>
</tr>
<tr>
<td>% late apoptotic CD3+ Lymphocytes</td>
<td>3.63 (1.68-9.00)</td>
<td>3.49 (1.53-11.27)</td>
<td>ns</td>
<td>3.59 (1.47-8.40)</td>
<td>ns</td>
</tr>
<tr>
<td>Periperal blood cell counts (N=16)</td>
<td></td>
<td></td>
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<tr>
<td>Lymphocytes (x 10^9 cells/L)</td>
<td>1.32 (1.21-2.20)</td>
<td>1.34 (1.24-2.14)</td>
<td>0.044</td>
<td>1.65 (1.30-2.48)</td>
<td>0.013</td>
</tr>
<tr>
<td>Monocytes (x 10^9 cells/L)</td>
<td>0.63 (0.35-0.79)</td>
<td>0.58 (0.36-0.77)</td>
<td>ns</td>
<td>0.54 (0.34-0.77)</td>
<td>ns</td>
</tr>
<tr>
<td>Neutrophils (x 10^9 cells/L)</td>
<td>4.60 (2.81-6.00)</td>
<td>4.26 (2.86-5.81)</td>
<td>ns</td>
<td>4.09 (2.77-5.37)</td>
<td>ns</td>
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Cell lysis and complement activation

<table>
<thead>
<tr>
<th>LDH (U/L) (N=16)</th>
<th>171 (145-216)</th>
<th>162 (138-205)</th>
<th>0.027</th>
<th>187 (156-219)</th>
<th>ns</th>
</tr>
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<tbody>
<tr>
<td>CRP (mg/L) (N=16)</td>
<td>8.3 (2.2-41.1)</td>
<td>8.4 (2.0-40.0)</td>
<td>0.047</td>
<td>7.7 (2.2-36.8)</td>
<td>ns</td>
</tr>
<tr>
<td>C4b/c levels (nM) (N=10)</td>
<td>11.5 (8.0-14.0)</td>
<td>11.5 (9.0-13.0)</td>
<td>ns</td>
<td>10.0 (6.6-18.0)</td>
<td>ns</td>
</tr>
</tbody>
</table>

*Data are presented as median and interquartile range. P values < 0.05 (exact significance, two-tailed) are shown in Italic. Time points represent before (T=0), 1 hour (T=1) and 24 hours (T=24) after infliximab infusion. Data at 1 and 24 hours, respectively, compared with baseline values.

Decreased numbers of synovial T cells and macrophages 24 hours after the infliximab infusion.
Twenty-four hours after infliximab treatment we observed a slight increase in TUNEL + cells in 3 out of 5 patients with a decrease in 2 patients (Figure 2B-F). An increase in active caspase 3 cells was seen in only one patient. Interestingly, electron microscopy demonstrated no apoptotic cells at any of the time points (Figure 3A-C), with the exception of one patient after 24 hours infliximab treatment, at which time point apoptotic macrophages were detected (Figure 3D). This observation coincided with an increase in active caspase 3 as well as TUNEL + cells. In conclusion, in spite of the reduction in T cells and macrophages observed early after initiation of infliximab treatment in the present study and in a previous study (4), we found apoptotic cells in the synovium in only 1 patient at 24 hours.

Figure 2. Synovial Apoptosis. A and B, Number of TUNEL+ cells/mm² in paired synovial tissue sections obtained from 5 patients with rheumatoid arthritis (pat1–pat5) before treatment (T = 0) and 1 hour after infliximab treatment (A) and from 5 patients (pat6–pat10) before treatment and 24 hours after treatment (B). There was a minor increase in the number of TUNEL + cells/mm² in 2 patients and a decrease in 3 patients 1 hour after treatment. There was an increase in the number of TUNEL+ cells in 3 patients 24 hours after treatment. In 1 patient only (patient 9) this was associated with an increase in the number of active caspase 3+ cells and the number of apoptotic cells, determined by electron microscopy. None of the differences were statistically significant. C–F, Staining for TUNEL cells (purple staining) in paired synovial tissue sections obtained from 2 patients before treatment (C and E) and 24 hours after treatment (D and F). No increase in the number of TUNEL+ cells was seen in 2 of 5 patients (C and D). A slight increase in the number of TUNEL+ cells was seen in 3 of 5 patients 24 hours after the first infusion (E and F). See color figures page 216.
Reduced VCAM-1 expression very early after infliximab treatment.

One hour after the infliximab infusion there was a trend towards lower VCAM-1 expression (median 409.006 IOD/mm², IQR 109.392-1048.080 before and 320.376 IOD/mm², IQR 164.912-586.039 1 hour after treatment), whereas ICAM-I expression was unaltered at that time point (median 47.677 IOD/mm², IQR 14.761-925.666 before and 48.998, IQR 23.443-358.723 IOD/mm² 1 hour after treatment). Consistent with these data, there was reduced VCAM-I expression 24 hours after the infliximab infusion (589.652 IOD/mm², IQR 169.258-1073.837 before to 261.450 IOD/mm², IQR 73.442-432.562, 24 hours after treatment). There was not sufficient tissue to evaluate ICAM-I expression at that time point. In spite of the clear trend for VCAM-1 expression, none of the changes were statistically significant, presumably due to the relatively small number of patients.

DISCUSSION

The results presented here show that the decrease in numbers of macrophages and T cells observed in the rheumatoid synovium early after the first infliximab infusion cannot be explained by apoptosis induction at the site of inflammation. Moreover, there are no signs of apoptosis induction in peripheral blood mononuclear cells within the first 24 hours of
TNF blocking therapy and apoptosis induction

1. treatment. Together, this study supports the previously proposed hypothesis that in vivo anti-
2. TNF treatment reduces synovial inflammation primarily by reducing cell influx and promoting
3. cell efflux rather than by induction of apoptosis at the site of inflammation. However, the results
4. do not exclude the possibility that after more prolonged treatment the synovial cells become
5. susceptible to apoptosis secondary to the decrease in inflammation, as could occur after any
6. anti-inflammatory treatment (23).
7.
8. Previous studies on the effects of anti-TNF antibodies on peripheral blood mononuclear cells
9. have shown variable results in different cell populations under diverse experimental conditions.
10. One study for instance demonstrated apoptosis induction in peripheral blood monocytes from
11. patients with Crohn's disease after in vitro treatment with infliximab (24). Another study found
12. apoptosis induction in CD3/CD28-stimulated peripheral blood T lymphocytes from normal
13. individuals after in vitro treatment with infliximab, but not etanercept (25). However, another
14. report described a non-significant increase in apoptosis in peripheral blood monocytes of RA
15. patients after in vitro incubation with either infliximab or etanercept, without any effect on
16. peripheral blood T lymphocytes (9). Collectively, the results appear highly dependent on the
17. experimental conditions.
18.
19. To investigate whether apoptosis induction in peripheral blood mononuclear cells after anti-
20. TNF antibody therapy is relevant in vivo, we analyzed circulating monocytes and T cells from
21. RA patients during the 24 hours following infliximab infusion. This time point was chosen,
22. since a marked decrease in inflammation can be observed as early as 48 hours (4) after the
23. first infliximab infusion. Of importance, there was no reduction in peripheral blood monocytes
24. or T cells, no increase in the number of apoptotic cells in the peripheral blood, no elevation in
25. nucleosome levels nor signs of cell lysis or complement activation. The lack of increase in the
26. number of apoptotic cells in the blood is in agreement with similar findings in patients with
27. Crohn's disease who were treated with infliximab (26).
28.
29. Under normal conditions nucleosomes are released by late apoptotic cells (17); in contrast to a
30. previous study in a group of 11 patients with a mixture of rheumatological diseases, suggesting
31. an increase in nucleosome levels detectable at 2 hours and 24 hours after infliximab treatment
32. (27), we did not detect any circulating nucleosome levels in the 21 RA patients within the first
33. 24 hours after infliximab treatment. It is difficult to explain the discrepancy, but our data were
34. consistent using 2 independent assays. Obviously, it is conceivable that there may be elevated
35. nucleosome levels after more prolonged treatment, resulting from a change in the balance
36. between pro- and anti-apoptotic signals secondary to reduced inflammation, but we did not
37. find any evidence to support apoptosis induction as a primary mechanism of action of anti-TNF
38. therapy.
39.
Since opsonization of apoptotic cells, by direct binding of complement or complement binding through CRP, accelerates clearance of these cells through phagocytosis (28), we also measured activated C4 (C4b/c) levels as a reflection of activation of the classical complement pathway. We did not find an increase in C4b/c levels within the first 24 hours after infliximab treatment. These data suggest that complement-dependent cytotoxicity does not occur in vivo in the first day after the infliximab infusion. Since CRP levels did not decrease 24 hours after treatment we can assume that opsonization of apoptotic cells by CRP (29) and subsequent phagocytosis is not hampered at this early time point. Moreover, there were no signs of cell lysis reflected by a change in LDH concentrations, consistent with previous observations (27). Together, we conclude that there were no signs of apoptosis induction or cell lysis in peripheral blood mononuclear cells that could explain the reduction in inflammation early after infliximab administration.

Our previous work has suggested that reduced synovial inflammation detected as early as 48 hours after initiation of infliximab treatment does not result from apoptosis induction at the site of inflammation (4;7). In the present study we extend these findings by showing a reduction in median numbers of synovial T cells and macrophages 24 hours after the first infusion with infliximab. A limitation of this part of the study is the restricted number of patients; it is difficult, however, to perform intensive studies involving 2 arthroscopies in a 24 hour period in larger numbers of patients. Similar to what we have found before at the 48 hour time point, we did not detect evidence of apoptosis induction in the synovium at either 1 hour after completion of the infusion or at 24 hours, except in one patient at 24 hours. This patient could not be distinguished from the other patients on the basis of clinical features.

The lack of apoptosis induction in the tissue appears to differ from results obtained in patients with Crohn’s disease: 24 hours after the infliximab infusion an increase in the number of TUNEL positive T cells was found in the lamina propria (12). It is possible that this difference relates to the different dosage used (3 mg/kg in the present study versus 5 mg/kg in patients with Crohn’s disease) or to the particularly anti-apoptotic status of the RA synovium (8). It should also be noted that in patients with Crohn’s disease indirect evidence for apoptosis induction has been shown by TUNEL assays (12) and annexin V scans (30), but no definite evidence by electron microscopy. The recent experience with certolizumab, a pegylated Fab’ fragment of a humanized anti-TNF-alpha antibody that is not able to induce apoptosis, supports the notion that apoptosis induction is not only not required to induce improvement in RA, but also not in Crohn’s disease (31).

If the marked decrease in synovial inflammation early after initiation of anti-TNF antibody treatment is not the result of apoptosis induction, how could we explain these changes at the site of inflammation? Previous studies have shown that TNF blockade results in a reduction in...
the expression of adhesion molecules (5;32), and chemokines (6), molecules that are intimately involved in cell migration and retention at the site of inflammation. Thus, TNF blockade may directly block the migration of inflammatory cells to the inflamed synovial compartment and might also enhance cell egress. Consistent with this notion is the previously reported reduction in neutrophils trafficking to the inflamed joint after TNF blockade, as shown by radionucleide scans (6).

Further support for the hypothesis of a block in cell influx comes from the observation that there was an increase in circulating lymphocytes directly after initiation of infliximab treat-

ment as shown in the present study as well as in previous studies (32-34). Moreover, we found decreased VCAM-1 expression very early after the infliximab infusion. Recent work suggests that promotion of lymphangiogenesis after TNF blockade could also play a role in promoting efflux from the inflamed tissue (35). In conclusion, the data presented here support the hypothesis that in RA neutralization of TNF, resulting in decreased expression of adhesion molecules and chemokines (5;6;32) with a subsequent reduction in migration and retention of inflammatory cells, might be sufficient to induce clinical improvement.

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TNF blocking therapy and apoptosis induction


